CD49d promotes disease progression in chronic lymphocytic leukemia: new insights from CD49d bimodal expression

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Key Points:

-CLLs with bimodal CD49d expression account for ~20% of CLL and display distinct clinico-biological features.

-The prognostic impact of CD49d is increased by considering CD49d bimodal expression.

Abstract

CD49d is a remarkable prognostic biomarker of chronic lymphocytic leukemia (CLL). The extensively validated 30% of positive CLL cells cut-off value is able to separate CLL patients into two subgroups with different prognosis, but it does not consider the pattern of CD49d expression. In the present study, we analysed a cohort of 1,630 CLL samples and identified the presence of ~20% of CLL cases (n=313) characterized by a bimodal expression of CD49d, i.e. concomitant presence of a $CD49d_{pos}$ sub-population and a $CD49d_{neg}$ sub-population. At variance with the highly stable CD49d expression observed in CLL patients with a homogeneous pattern of CD49d expression, CD49d bimodal CLL showed a higher level of variability in sequential samples, and an increase in the $CD49d_{pos}$ sub-population over time after therapy. The $CD49d_{pos}$ sub-population from CD49d bimodal CLL displayed higher levels of proliferation compared to the $CD49d_{neg}$ cells, was more highly represented in the bone marrow compared to peripheral blood (PB), and in PB CLL subsets expressing the $CXCR4_{dim}/CD5_{bright}$ phenotype, known to be enriched in proliferative cells. From a clinical standpoint, CLL patients with CD49d bimodal expression, regardless of whether the $CD49d_{pos}$ sub-population exceeded or not the 30% cut-off, experienced a clinical behavior similar to $CD49d_{pos}$ CLL, both in the chemo-immunotherapy (n=1,522) and in the ibrutinib (n=158) settings. Altogether, these results suggest that CD49d can drive disease progression in CLL, and that the pattern of CD49d expression should be also considered to improve the prognostic impact of this biomarker in CLL.

Introduction

CD49d, the rate-limiting alpha chain of the CD49d/CD29 integrin heterodimer very late antigen-4 (VLA-4), expressed in ~40% of chronic lymphocytic leukemia (CLL) cases, is a strong independent predictor of survival and treatment need in CLL.[1-6]

CD49d/CD29 mediates both cell-cell and cell-matrix interactions in CLL-involved tissues, delivering pro-survival signals and protecting CLL cells from drug-induced apoptosis.[7] Moreover, CD49d/CD29 has a key role in driving homing of CLL cells to lymphoid tissues.[8,9] This is confirmed by the association between high CD49d expression and the presence of lymphadenopathy at diagnosis, the development of lymphadenopathy during the course of the disease, and the almost universal CD49d expression in CLL subsets with prevalent nodal disease.[10-12] Lymph-nodes (LNs) represent the predominant sites of CLL cell activation and proliferation.[13,14] Concordantly, LN-derived CLL cells are characterized by a distinct gene expression profile and phenotype, including higher CD49d expression than in paired peripheral blood (PB) samples.[8,15] A higher CD49d expression was also documented in PB $CXCR4_{low}/CD5_{bright}$ CLL cells, a cell subset representing the proliferative LN-derived CLL cell fraction.[8,16]

In CLL, CD49d expression is variable, from completely negative cases to cases uniformly expressing the molecule at very high levels.[17-19] The 30% cut-off, which robustly separates CLL patients into two subgroups with different prognosis, has been extensively validated.[1-5] However, the simple use of the cut-off does not provide information on the expression pattern of CD49d. Indeed, we previously observed that CD49d may be expressed in a bimodal pattern in CLL, with the co-presence of clearly distinct negative and positive groups of cells in the same sample.[20,21] In this context, $CD49d_{pos}$ sub-populations, especially when represented in <30% of cells, raises the question whether they may or may not impact disease progression.

Here, we analysed CD49d expression in a large cohort of CLL cases with the following aims: i) determine the exact frequency of CLL cases with a bimodal pattern of CD49d expression; ii) highlight potential functional differences between the $CD49d_{neg}$ and $CD49d_{pos}$ cells in CLL expressing CD49d with a bimodal pattern; iii) evaluate the clinical impact of CD49d bimodal expression.

Methods

CLL patients and primary CLL cell characterization

The study, performed under the IRB approval of the Aviano Centro di Riferimento Oncologico (Approvals n. IRB-05-2010 and n. IRB-05-2015), included 1,630 CLL diagnosed and treated according to the current iwCLL guidelines,[22] from a consecutive series of 2,045 multicenter patients all referred to the Clinical and Experimental Onco-Hematology Unit of the Centro di Riferimento Oncologico in Aviano for immunocytogenetic analyses between 2006 and 2017. For the purposes of clinical analyses, patients were split in two cohorts: cohort A (764 patients from a single center) and cohort B (866 patients from 5 different centers), whose clinical and biological features are summarized in Supplemental Table 1. This study also included 158 CLL patients (47 cases relapsed/refractory [RR] CLL from the cohort of 1,630, and additional 88 RR and 23 treatment-naïve [TN] CLL) treated with ibrutinib in the context of a multi-center Italian named patient program (NPP); clinical and biological features of this cohort are summarized in Supplemental Table 2. Informed consent was obtained in accordance with the Declaration of Helsinki. Patients' characterization included *IGHV* mutational status, cytogenetic abnormalities, *TP53* and *NOTCH1* mutations, all detected as previously reported,[2,23-27] and clinical parameters, such as age, Rai stage, beta-2-microglobulin (B2M) levels.

Immunophenotypic analyses, cell sorting, proliferation assay and telomere length measurements

Immunophenotypic analyses and cell sortings were performed on fresh peripheral blood (PB), or paired PB and bone marrow (BM) samples at different time points utilizing a FACSCantoI and a FACSAriaIII (BD Biosciences, La Jolla, CA, USA) flow cytometer/cell sorter upon instrument calibration with CS&T beads (BD Biosciences) using the FACSDiva software (BD Biosciences). Detailed guidelines for the analysis and definition of CD49d bimodal expression[28] are reported in Supplemental materials.

Proliferation and telomere length assays were performed using previously published procedures.[29,30] Details on these assays, and on the statistics utilized for clinical correlations are included in Supplemental materials.

Results

Frequency of CD49d bimodal expression in CLL

CD49d expression was analysed in PB samples from 1,630 CLL cases at diagnosis (Supplemental Table 1). According to the 30% cut-off identified for clinical purposes,[1,2] 904 cases (55.5%) were CD49d$_{neg}$ and 726 cases (44.5%) were CD49d$_{pos}$. In 313 cases (19.2%), CD49d had a bimodal expression (bimCD49d), with two separate cell populations, one completely negative, with a fluorescence signal superimposable to that of the negative control, and the other positive, characterized by a fluorescence signal completely above the cut-off (Figure 1A and Supplemental Figure 1). CLL cells from the remaining 1,317 patients (80.8%) expressed CD49d in a homogeneous pattern (homCD49d) (Figure 1B). In terms of percentage of CD49d-expressing CLL cells, virtually all values (ranging from 1% to 99%) were represented both in the bimCD49d and homCD49d group. However, while in bimCD49d cases all CD49d expression values were almost equally represented, 90% of CLL with homCD49d expression showed either very low (56% of cases with expression ≤20% CD49d) or very high (34% cases with expression ≥80% CD49d) expression levels, with as low as 2% of cases clustered around the 30% cut-off (Figure 1C and 1D), in keeping with previous observations.[1,2] Of note, the vast majority of cases expressing CD49d around the 30% cut-off (CD49d expression ranging between 20% and 40%) belonged to the bimCD49d group (67/94 cases, 71%).

The CD49d$_{neg}$ and CD49d$_{pos}$ cell sub-populations from bimCD49d CLL derive from the same clone

The *IGHV* mutational status was assessed by Sanger sequencing in 303 bimCD49d. In 283/303 (93.4%) cases, a unique *IGHV* sequence was detected, whereas among the remaining 20 cases either multiple *IGHV* sequences were found (n=14) or no *IGHV* sequence could be determined (n=6). A detailed description of the *IGHV* gene usage and status is reported in Supplemental Table 3. The *IGHV* sequence analysis was then repeated by NGS in the sorted CD49d$_{neg}$ and CD49d$_{pos}$ fractions from 31 bimCD49d samples with variable amount of CD49d$_{pos}$ sub-population, including one bi-clonal sample presenting two different immunoglobulin rearrangements by Sanger sequencing. NGS analysis confirmed a unique *IGHV* sequence in both CD49d$_{neg}$ and CD49d$_{pos}$ fractions in 30 cases, and the concomitant presence of two sequences in the bi-clonal sample, without differences between the CD49d$_{neg}$ and CD49d$_{pos}$ fractions (Supplemental Table 3).

The proportion of the CD49d$_{pos}$ sub-population in bimCD49d CLL tends to increase over time

Sequential samples were available in 94 bimCD49d and in 322 homCD49d patients (median sample interval: 34 months [range 3-116] for bimCD49d, and 35 months [range 6-161] for homCD49d). Forty-two (44.7%) and 131 (40.7%) patients from the bimCD49d and homCD49d groups respectively had received one line of therapy after the first sampling, whereas the remaining patients received no treatment before the two samplings.

Compared to homCD49d CLL, where CD49d expression was highly stable over time (Supplemental Figure 2AB), bimCD49d cases were characterized by a higher variability of CD49d expression between the first and the second sample (Supplemental Figure 2CD). Despite this, the proportion of CD49d$_{pos}$ cells, almost stable between the first and the second sample in untreated bimCD49d cases (Figure 2A), increased after the first line of treatment (p=0.03) (Figure 2B). Moreover, a detailed analysis of CD49d expression in sequential samples from additional bimCD49d CLL patients receiving two to four lines of therapy (n=13; median sample intervals= 33 months), highlighted an increasing proportion of CD49d$_{pos}$ cells with a tendency towards plateau as the number of treatment lines increased (Figure 2C).

The proportion of CD49d$_{neg}$ and CD49d$_{pos}$ cell sub-populations varies among different tissue compartments

The proportion of CD49d$_{neg}$ and CD49d$_{pos}$ sub-populations was analysed in paired PB and BM samples from 13 bimCD49d cases. A higher amount of CD49d$_{pos}$ cells was observed in BM compared to PB samples (p=0.0006). Notably, in 3 cases the CD49d$_{pos}$ subset increased from <30% in the PB to >30% in the BM (Figure 3A).

We next analysed the amount of CD49d$_{pos}$ cells in the context of subpopulations with variable reciprocal densities of CXCR4/CD5 (Supplemental Figure 3), representing cell subsets defined as "recent emigrants from tissue sites" (CXCR4$_{dim}$/CD5$_{bright}$) or "attempting to re-enter the tissue sites" (CXCR4$_{bright}$/CD5$_{dim}$)[16] in 147 bimCD49d CLL. A median of 8% (range 3-35%) and 7% (range 3-14%) of total CLL populations was identified as CXCR4$_{dim}$/CD5$_{bright}$ and CXCR4$_{bright}$/CD5$_{dim}$, respectively. Overall, the CXCR4$_{dim}$/CD5$_{bright}$ fractions were characterized by significantly higher proportions of CD49d$_{pos}$ cells compared to their paired CXCR4$_{bright}$/CD5$_{dim}$ fractions (p<0.0001) (Figure 3B).

CD49d$_{pos}$ cells display a higher propensity to proliferate than CD49d$_{neg}$ cells

The CXCR4$_{dim}$/CD5$_{bright}$ cell fraction is known to mark CLL subsets with increased proliferation compared to CXCR4$_{bright}$/CD5$_{dim}$ cells.[16] Consistently, proliferation experiments using cells from bimCD49d CLL (n=10), evidenced a higher proportion of proliferating cells in the CD49d$_{pos}$ compared to the CD49d$_{neg}$ fractions (p=0.004) (Supplemental Figure 4A-B), and no acquisition or loss of CD49d expression by the CD49d$_{neg}$ and CD49d$_{pos}$ fractions respectively (Supplemental Figure 5).

We next examined the telomere length in paired CD49d$_{neg}$ and CD49d$_{pos}$ sub-populations from 15 CLL samples (Supplemental Figure 6). Despite an intra-clonal variation in the length of individual telomeres, all sub-populations tested showed overall short telomeres, indicating that they had undergone a large number of cell divisions,[31] without substantial differences between the paired CD49d$_{pos}$ and CD49d$_{neg}$ cells (mean telomere length 3.1 kb and 3.2 kb, respectively). Of note, in two cases (CLL#7 and CLL#8, Supplemental Figure 6, arrows) the CD49d$_{pos}$ sub-populations displayed shorter telomeres (>1 kb difference) compared to the CD49d$_{neg}$ cells suggesting a greater proliferative history for the CD49d$_{pos}$ cell fractions.

Finally, we performed a comprehensive cytogenetic and mutation analysis in the sorted CD49d$_{neg}$ and CD49d$_{pos}$ cell fractions from 56 bimCD49d CLL cases characterized by one or more genetic abnormalities (75 total tests).[4,32] In this context, significant associations between CD49d$_{pos}$ cells with trisomy 12 and *NOTCH1* mutations, as previously described by us,[20,21] and between CD49d$_{neg}$ cells and 17p deletion, were detected (Supplemental Tables 4-5).

CLL patients with CD49d bimodal expression have a clinical behavior similar to CD49d$_{pos}$ CLL in the context of standard chemo-immunotherapy

To study the clinical behavior of bimCD49d CLL in terms of overall survival (OS), CLL cases were split in two homogeneous cohorts (cohort A and cohort B, see Methods and Supplemental Table 1). To exclude a potential bias due to improved survival for patients treated with novel agents including ibrutinib, only patients treated with standard chemo-immunotherapy were included in these analyses (n= 1,522).

Notably, when comparing the clinical outcomes of bimCD49d$_{neg}$ (i.e. with a CD49d$_{pos}$ component <30% cut-off) and bimCD49d$_{pos}$ (i.e. with a CD49d$_{pos}$ component ≥30% cut-off) CLL, no OS differences were found in the separate and combined cohorts (Supplemental Figure 7A-B and

Figure 4A). Moreover, no other cut-off values able to split bimCD49d cases into two groups with different OS could be selected by ROC curve analyses (Supplemental Figure 8A-B). Conversely, the main clinical and biological parameters showed the expected trends of OS in both cohorts (Supplemental Figure 9). Given the superimposable clinical behavior of bimCD49d$_{neg}$ and bimCD49d$_{pos}$ CLL, we merged them in a single group and compared their OS probabilities with those of homCD49d CLL split according to the 30% cut-off. BimCD49d cases displayed significantly shorter OS compared to homCD49d$_{neg}$ CLL ($p<0.0001$ for cohort A, $p=0.0013$ for cohort B, and $p<0.0001$ for the combined cohort), and no difference with homCD49d$_{pos}$ CLL in both the separate and combined cohorts (Supplemental Figure 7C-D and Figure 4B).

The same analyses were performed using the treatment-free survival (TFS) readout. The results obtained, overall similar to those obtained using the OS readout, are summarized in Supplemental Figures 10-12.

CD49d bimodal expression has independent prognostic impact in patients treated with standard chemo-immunotherapy

To test the independent prognostic impact of bimCD49d expression, patients were split in three groups: homCD49d$_{neg}$, homCD49d$_{pos}$ and bimCD49d. As summarized in Supplemental Table 6, the frequency of various negative clinical and biological features in bimCD49d CLL was usually intermediate between that observed in homCD49d$_{neg}$ and homCD49d$_{pos}$ CLL, with the exclusion of del17p/*TP53*disruption that showed the lowest frequency in bimCD49d CLL. By univariable analysis, both homCD49d$_{pos}$ and bimCD49d had a significantly increased hazard of death (HR=3.18, 95% CI 2.41-4.19, and HR=2.37, 95% CI 1.71-3.3, respectively) compared to homCD49d$_{neg}$ patients (HR= 1.0, reference; Table 1). By Cox analysis for OS, both homCD49d$_{pos}$ and bimCD49d remained independent predictors after adjusting for Rai stage, age, *IGHV* mutation status, del17p, del11q, *TP53* and *NOTCH1* mutation status in a model that not included B2M (Table 1, model I, n=1,045) or included B2M (Table 1, model II, n=753). The same was observed in multivariable models considering *TP53* disruption instead of del17p and *TP53* mutations (Supplemental Table 7).

Comparable results were obtained in a Cox analysis for TFS. Again, homCD49d$_{pos}$ and bimCD49d had an increased risk of being treated in models that either included B2M (n=831) or not included B2M (n=1,140) (Supplemental Table 8).

Finally, we compared the prognostic power of CD49d considered either as dichotomous according to the canonical 30% cut-off, or merging homCD49d$_{pos}$ plus bimCD49d CLL, both in univariable analysis and in the context of multivariable analyses that included Rai stage, age, *IGHV* and *TP53* mutation status (Supplemental Table 9). In both analyses, merging homCD49d$_{pos}$ and bimCD49d CLL outperformed the 30% cut-off (C-index=0.63, 95% CI 0.59-0.67 vs 0.64, 95% CI 0.60-0.67, p<0.001) and improved the prognostic power of the Cox model (C-index=0.799, 95% CI 0.748-0.809 vs 0.803, 95% CI 0.750-0.814, p<0.001). Of note, bimCD49d cases that would have been mis-assigned to a low risk group accounted for 9% of cases, corresponding to 147 cases in our cohort.

CD49d bimodal expression correlated with shorter progression-free survival in ibrutinib treated patients

The impact of CD49d bimodal expression on progression-free survival (PFS) under ibrutinib was evaluated in a cohort of 158 cases treated with ibrutinib in the context of an Italian multicenter NPP, with a median follow-up of 16 months (Supplemental Table 2). Among 158 patients, 29 were from a previous study of ours,[33] with follow-up information here updated. In all cases, CD49d expression was evaluated before starting ibrutinib.

The ibrutinib cohort was characterized by 39 (24.7%) bimCD49d and 119 (75.3%) homCD49d cases. Also in this context, the CD49d 30% cut-off split homCD49d but not bimCD49d CLL into two groups with different prognosis (Figure 4C and Supplemental Figure 13A). Moreover, bimCD49d cases displayed shorter PFS than homCD49d$_{neg}$ CLL (p=0.004), and no different PFS when compared to homCD49d$_{pos}$ CLL (Figure 4C). Consistently, homCD49d$_{pos}$ and bimCD49d CLL combined in a single group showed reduced PFS compared to homCD49d$_{neg}$ CLL (p= 0.0052, Supplemental Figure 13B).

The prognostic impact of homCD49d$_{pos}$/ bimCD49d CLL was then tested in multivariable analyses. HomCD49d$_{pos}$/ bimCD49d CLL retained independent negative prognostic impact together with *TP53* disruption and >1 previous therapies, both in a multivariable model that considered the whole cohort of ibrutinib-treated patients (n=158), and in a subgroup of R/R CLL (n=124) where information on other prognostic factors,[34] was available (Table 2).

We then verified if the proportion of CD49d$_{pos}$ cells varied over time during ibrutinib treatment in patients with bimCD49d expression. Two consecutive samples were available in 8 bimCD49d

cases, with a median time between the pre-ibrutinib and post-ibrutinib sample collection of 28 months (range 8-50). In all cases, an increased proportion of $CD49d_{pos}$ cells emerged after treatment (p=0.0078, Figure 4D and Supplemental Figure 14).

CD49d expression and CLL-IPI risk categories

We finally tested if CD49d expression had an impact on overall survival prediction in the context of the risk categories identified by the international prognostic index (CLL-IPI).[35] Complete data to score CLL cases according to the CLL-IPI were available in 878 patients from our cohort. The CLL-IPI was able to efficiently segregate CLL patients in four risk categories characterized by significantly different OS (Figure 5A). In the context of each risk category, we further split patients into two groups according to CD49d expression, considering $homCD49d_{pos}$ and bimCD49d CLL as a single group ($CD49d_{pos}$/bimCD49d). $CD49d_{pos}$/bimCD49d CLL showed reduced OS in all CLL-IPI risk categories with the exception of the very high risk group, where the survival probability of the very few $CD49d_{neg}$ patients (n=16) was similar to that of $CD49d_{pos}$/bimCD49d patients (Figure 5B-E).

Discussion

This study was set out to investigate the functional and clinical implications of the bimodal expression of CD49d in CLL. The presence of CLL characterized by bimodal CD49d expression had been indicated in previous studies by us,[20,21] but the frequency and the clinical impact of CD49d bimodal expression has never been addressed. Here, using a large cohort of patients, we showed that the frequency of CD49d bimodal cases accounted for ~20% of total cases. The $CD49d_{neg}$ and $CD49d_{pos}$ sub-populations from the large majority of these bimodal cases shared the same *IGHV* rearrangement, confirming a common clonal origin.

CD49d in CLL is expressed at either very low or very high levels in most cases, with few cases displaying expression levels around the 30% cut-off. [1,2] This characteristic, associated with a low probability of misclassifying patients, is further emphasized by the results from the present study. Indeed, after sorting out CLL cases with CD49d bimodal expression, the frequency of CLL cases with 20-40% CD49d expression in the homCD49d group turned out to be even lower (from 6% to 2%, i.e. 27/1,630, cases).

The temporal stability, another important feature of CD49d expression,[1,2,36] has been here confirmed, particularly in the context of CLL completely lacking CD49d, in line with the methylation-dependent regulation of CD49d expression.[21] On the other hand, a fine-tune regulation of CD49d expression, observed in CLL cases with moderate to high CD49d expression, has been already reported in previous studies showing that microenvironmental stimuli are able to increase CD49d expression.[20,37] Since our flow cytometry analyses, based on the acquisition of at least 10,000 CLL events, could reach the 1% sensitivity, we cannot exclude the presence of lower percentages of $CD49d_{pos}$ cells in CLL grouped as hom$CD49d_{neg}$. However, in none of these cases with sequential samples available, a measurable $CD49d_{pos}$ clone emerged. At variance with what reported in homCD49d CLL cases, bimCD49d cases showed an overall increase of the $CD49d_{pos}$ sub-population after treatment, with a tendency towards a plateau subsequent to multiple lines of therapy. This observation may be explained by the selection of $CD49d_{pos}$ CLL cells that find protection from therapy and/or gain proliferative advantage in the context of microenvironmental niches.[38] Consistently, the $CD49d_{pos}$ sub-population of bimodal cases was increased in the BM, which is a privileged site of relapse in CLL,[39,40] compared to PB. Even though we had not the chance to analyse paired LN-derived samples from CD49d bimodal cases, the analysis of CD49d expression in the context of the proliferative fraction $CXCR4_{low}/CD5_{bright}$, which was indicated as the group of cells recently egressed from lymphoid tissues,[16] highlighted a higher proportion of

CD49d$_{pos}$ cells compared to their paired fractions. Consistently, bimCD49d cases presented with LN involvement at diagnosis more frequently compared to homCD49d$_{neg}$ CLL, but less frequently than CD49d$_{pos}$ CLL, where baseline lymphadenopathy accounted for ~62% of cases, in line with previously reported data.[10] In this context, it remains to be determined if bimCD49d CLL may be more prone than homCD49d$_{neg}$ CLL to develop lymphadenopathy during the course of the disease.[10]

In keeping with the association with a CXCR4$_{low}$/CD5$_{bright}$ phenotype,[16] CD49d$_{pos}$ sub-populations also showed a higher proliferation potential than the CD49d$_{neg}$ counterpart. The reasons behind this phenomenon cannot be explained by the mere expression of CD49d, but rather can be sought in the preferential expression by the CD49d$_{pos}$ cells of other co-stimulatory molecules. Even though the present study has not addressed this issue, previous reports by our and other groups showed high correlation and close relationship between expression of CD49d and CD38,[19,41-43] a molecule that was reported to label a subset enriched in proliferating cells within CLL clones, and associated with the risk of disease progression even when expressed in a sub-fraction of CLL cells.[44,45] Moreover, here we corroborated our previous findings of a significant association between CD49d$_{pos}$ cells with trisomy 12 and *NOTCH1* mutations,[26,27] pointing to a possible role of *NOTCH1* mutations in driving cell survival and proliferation in CD49d$_{pos}$ cells through NF-kB pathway activation.[20,37]

Despite the higher proliferative capacity of the CD49d$_{pos}$ sub-populations observed in-vitro, CD49d$_{neg}$ and CD49d$_{pos}$ cells did not show different replicative histories in the majority of the investigated cases, pointing to a balanced proliferation rate *in-vivo*. However, a different telomerase activity between CD49d$_{neg}$ and CD49d$_{pos}$ cells can be hypothesized,[46] and it cannot be ruled out that a different telomere shortening may occur in follow-up samples, as it was reported in CLL cases with clonal evolution.[47]

In light of all previous considerations, it was not completely unexpected that bimCD49d CLL patients, even in the presence of a small CD49d$_{pos}$ sub-population, followed a clinical outcome similar to that of homCD49d$_{pos}$ patients in CLL cases treated with conventional chemo-immunotherapy. Of note, both homCD49d$_{pos}$ and bimCD49d expression retained independent prognostic impact in multivariate models which included the main clinical and biological prognosticators.[22] Consistently, the combination of homCD49d$_{pos}$ with bimCD49d CLL in one single group outperformed the 30% cut-off as OS predictor both in univariable and multivariable analyses.

The negative prognostic impact of CD49d in the ibrutinib setting, was previously reported by us in CLL patients from two independent ibrutinib-treated cohorts.[33] In the present study, we could confirm CD49d expression as a negative prognostic marker in patients treated with ibrutinib, and showed that both homCD49d$_{pos}$ CLL, and bimCD49d CLL experienced significantly shorter PFS than CD49d$_{neg}$ CLL. Moreover, the homCD49d$_{pos}$ and bimCD49d categories turned out both independent prognosticators in the context of multivariable models that included the main clinical and biological covariates with a clinical impact in the ibrutinib setting.[34] Further validation studies with longer follow-up and larger cohorts of patients are needed to definitely validate CD49d as OS predictor in the context of target therapies.

Our previous study showed that, after one year of ibrutinib treatment, the lymph node mass reduction was lower in CD49d$_{pos}$ compared to CD49d$_{neg}$ CLL.[33] Consistently, the increasing proportion of the CD49d$_{pos}$ sub-population observed here in eight CLL cases with CD49d bimodal expression after ibrutinib treatment, strongly supports the hypothesis of a higher resistance to the treatment for CD49d expressing cells. In the light of these data it would be interesting to evaluate whether higher percentages of the CD49d$_{pos}$ sub-population could be found in tissue compartments other than PB.

Overall, our data point to a potential relevant role of CD49d to clinical practice, also in the light of its ability to separate patients with different OS probability in the context of the CLL-IPI risk categories.[35] In this regard, further validation studies are needed to test whether including the CD49d expression, either homogeneous or bimodal, in a comprehensive prognostic risk score may help to better stratify patients treated with chemoimmunotherapy or targeted therapy.

In summary, in the present study we have reported for the first time the presence, among CLL, of ~20% of cases characterized by CD49d bimodal expression. The CD49d$_{pos}$ cells from bimodal CD49d CLL displayed higher levels of proliferation compared to the CD49d$_{neg}$ cells, and were more highly represented in the BM compared to PB, and in CLL subsets enriched in proliferative cells. The higher proliferative capacity of CD49d$_{pos}$ cells was in keeping with the tendency of the CD49d$_{pos}$ sub-population to increase over time especially after therapy. In this regard, it is tempting to speculate that the CD49d$_{pos}$ sub-population, even if it represents a minority of the tumor, may support the progression of the entire clone. Indeed, from a clinical point of view, the presence of small cell CD49d$_{pos}$ populations consistently associated with bad prognosis, suggesting to merge

together bimCD49d and hom$CD49d_{pos}$ CLL to improve the prognostic power of CD49d in the clinical setting.

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Authorship Contributions:

A.Z. and V.G. designed the study, coordinated the experiments and wrote the manuscript; E.T., F.P. and D.B performed immunophenotypic analyses, analysed the data, and contributed to write the manuscript; C.C., T.B., F.M.R., R.B., performed cell sorting, proliferation experiments and contributed to molecular characterization of the samples, P.N., H.C., I.C., and E.Z. performed immunophenotypic and molecular analyses, K.N. performed telomere length analyses, J.P. and G.T. performed statistical analyses, M.G., R.M., E.S., I.I., J.O., G.D.A., L.L., F.Z., G.P., A.C., F.D.R., D.R., G.G., and G.D.P. provided well characterized biological samples and contributed to paper revision; C.P. and T.N.H. contributed to scientific discussion, data interpretation and paper revision.

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Figure Legends

Figure 1. CD49d bimodal and homogeneous expression. (A-B) Histogram plots of CD49d expression (red) in two representative CLL cases with CD49d bimodal expression (A) and in two representative CLL cases with CD49d homogeneous expression (B). Grey histograms refer to unstained cells; (C-D) Relative percent frequency of CD49d expression in CLL with CD49d bimodal (C) and CD49d homogeneous (D) expression.

Figure 2. CD49d expression over time. CD49d expression was evaluated in two sequential samples from 94 CLL cases with CD49d bimodal expression either untreated (n=52, A) or treated once between the samples (n=42, B); (C) Variation of the amount of $CD49d_{pos}$ cells in sequential samples from 13 CD49d bimodal CLL treated with 2 lines of therapy (n=3, dark symbols), 3 lines of therapy (n=6, green symbols) or 4 lines of therapy (n=4, red symbols); p values refer to the paired Wilcoxon test (A-B) and to the Bonferroni-corrected Student's t-test (alpha=0.0125).

Figure 3. Amount of $CD49d_{pos}$ cells in different tissue compartments and in intra-clonal populations from bimCD49d CLL. (A) Percent of $CD49d_{pos}$ cells in paired peripheral blood (PB) and bone marrow (BM) samples from 13 CLL cases with CD49d bimodal expression; (B) Amount of $CD49d_{pos}$ cells in intra-clonal sub-populations with variable reciprocal densities of CXCR4/CD5 expression ($CXCR4_{dim}/CD5_{bright}$ and $CXCR4_{bright}/CD5_{dim}$ fractions) from 147 CLL cases with CD49d bimodal expression; p values refer to the paired Wilcoxon test.

Figure 4. Clinical impact of CD49d bimodal expression in the context of chemoimmunotherapy and ibrutinib treatment. (A) Overall survival Kaplan-Meier curves of all bimCD49d CLL cases split in $bimCD49d_{neg}$ (grey curves) and $bimCD49d_{pos}$ (black curves) groups according to the 30% cut-off; (B) Overall survival Kaplan-Meier curves of CLL cases with bimodal CD49d expression (bimCD49d, red curves), homogeneous negative ($homCD49d_{neg}$, grey curves) and homogeneous positive ($homCD49d_{pos}$, black curves) CD49d expression; (C) Progression-free survival Kaplan-Meier curves of ibrutinib-treated CLL cases with bimodal CD49d expression (bimCD49d, red curve), neg (grey curve) and pos (black curve) homogeneous CD49d expression (homCD49d). (D) CD49d expression in two sequential samples from 8 CLL cases with CD49d bimodal expression treated with ibrutinib between samplings; p values refer to the log-rank test (A-C) and to the paired Wilcoxon test (D).

Figure 5. Clinical impact of CD49d expression in the context of CLL-IPI categories. (A) Overall survival Kaplan-Meier curves of CLL cases split in the four risk categories of the CLL international prognostic index; (B-E) Overall survival curves of CLL cases with homogeneous negative CD49d expression (homCD49d$_{neg}$, grey curve) and the merging of homogeneous positive (homCD49d$_{pos}$) and bimodal CD49d (bimCD49d) expression (black curve) in the context of the low risk (B), intermediate risk (C), high risk (D) and very high risk (E) categories of the CLL-IPI; p values refer to the log-rank test.

Table 1. Cox regression analysis of overall survival.

Factor	Univariable analysis				Multivariable analysis (n=1,045) model I			Multivariable analysis (n=753) model II		
	cases	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*
homCD49d$_{pos}$*	1,522	3.18	2.41-4.19	<0.0001	2.13	1.52-2.99	<0.0001	2.65	1.76-3.98	<0.0001
bimCD49d†	1,522	2.37	1.71-3.30	<0.0001	2.12	1.44-3.12	0.0001	2.70	1.74-4.17	<0.0001
age >65	1,522	3.16	2.46-4.06	<0.0001	4.05	2.96-5.54	<0.0001	3.72	2.55-5.44	<0.0001
Rai stage II-IV	1,515	2.68	2.10-3.43	<0.0001	1.82	1.32-2.44	0.0001	n.i.	n.i.	n.i.
UM *IGHV*	1,385	3.88	3.02-5.00	<0.0001	2.41	1.79-3.24	<0.0001	2.78	1.99-3.91	<0.0001
del17p	1,407	4.81	3.29-7.03	<0.0001	n.i.	n.i.	n.i.	2.21	1.44-3.38	0.0003
del11q	1,407	2.56	177-3.69	<0.0001	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
tri12	1,407	1.82	1.29-2.56	0.0006	2.32	1.64-3.29	<0.0001	0.65	0.43-0.98	0.0423
TP53 mutated	1,253	3.04	2.23-4.16	<0.0001	2.41	1.79-3.24	<0.0001	n.i.	n.i.	n.i.
NOTCH1 mutated	1,444	2.02	1.54-2.65	<0.0001	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
β2M >ULN	1,017	4.12	3.01-5.63	<0.0001	-	-	-	1.73	1.18-2.52	0.0048

All factors used in univariable analyses were entered in the multivariable analysis;
*homCD49d$_{pos}$ refers to CLL cases with ≥30% homogeneous expression of CD49d; †bimCD49d refers to CLL cases with bimodal expression of CD49d.
Abbreviations: β2M, beta-2 microglobulin; CI, confidence interval; del, deletion; HR, hazard ratio; n.i., not included in the model after stepwise selection; tri, trisomy; ULN, upper limit of normal; UM, unmutated.

Table 2. Cox regression analysis for progression-free survival in ibrutinib-treated CLL.

	Whole ibrutinib-treated cohort (n=158)					
Factor	Univariable analysis			Multivariable analysis*		
	HR	95% CI	*p*	HR	95% CI	*p*
CD49d$_{pos}$†	3.03	1.34-6.87	0.0079	2.63	1.13-6.10	0.0242
UM *IGHV*	1.71	0.75-3.88	0.199	-	-	-
TP53 disruption	2.63	1.37-5.06	0.0038	2.10	1.06-4.14	0.0327
Rai stage III-IV	2.43	1.31-4.49	0.0046	n.i.	n.i.	n.i.
Lines of therapy >1‡	4.49	2.39-8.45	<0.0001	4.80	2.54-9.08	<0.0001
	R/R ibrutinib-treated CLL (n=124)					
Factor	Univariable analysis			Multivariable analysis§		
	HR	95% CI	*p*	HR	95% CI	*p*
CD49d$_{pos}$†	3.42	1.39-8.43	0.0075	3.41	1.32-8.79	0.0112
UM *IGHV*	1.94	0.67-5.58	0.2191	-	-	-
TP53 disruption	4.01	1.82-8.82	0.0006	3.06	1.34-6.97	0.0079
Hemoglobin <110 g/L for women <120 g/L for men	3.73	1.79-7.78	0.0004	-	-	-
Rai stage III-IV	2.87	1.40-5.91	0.0041	-	-	-
β2M ≥5 mg/L	1.09	0.44-2.68	0.8513	-	-	-
LDH >ULN	2.14	1.05-4.40	0.0373	-	-	-
Time from last therapy <24 months	1.98	0.90-4.38	0.0907	-	-	-
Lines of therapy >1‖	5.08	2.34-11.03	<0.0001	6.68	2.91-15.35	<0.0001

*All factors with a significant p value in univariable analyses were entered in the multivariable analysis;

† CD49d$_{pos}$ cases were obtained by combining cases with ≥30% homogeneous CD49d expression and cases with CD49d bimodal expression.

‡ comparison between 0-1 vs >1 lines of therapy.

§Significant features for multivariate analysis were selected through a Cox LASSO (least absolute shrinkage and selection operator) regression, selecting variables with non-zero coefficient under the best lambda selected by the model (Tibshirani R, Stat Med 1997; 16:385);

‖comparison between 1 vs >1 lines of therapy.

LDH, lactose dehydrogenase; all other abbreviations are explained in Table 1.

Figure 1

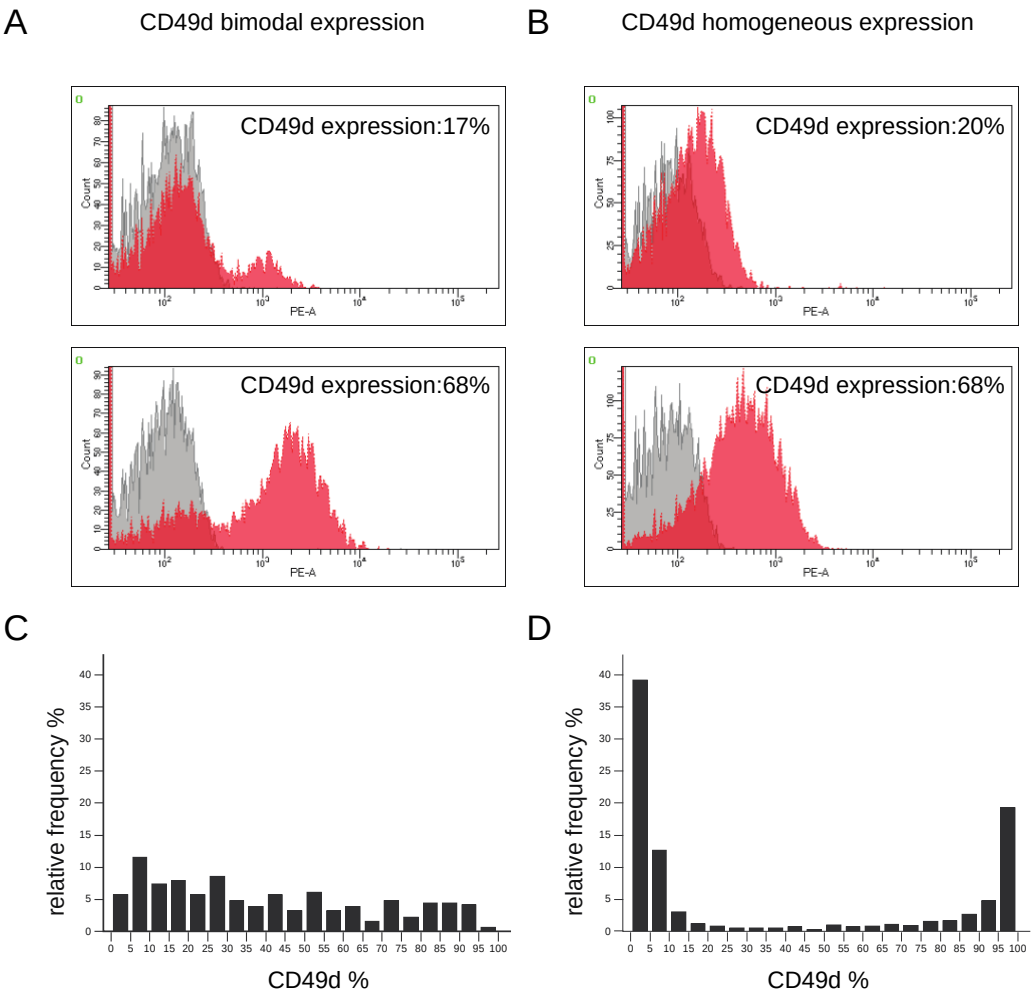

A
CD49d bimodal expression
B
CD49d homogeneous expression
CD49d expression:17%
CD49d expression:20%
CD49d expression:68%
CD49d expression:68%
Count
PE-A
C
D
relative frequency %
CD49d %

Figure 2

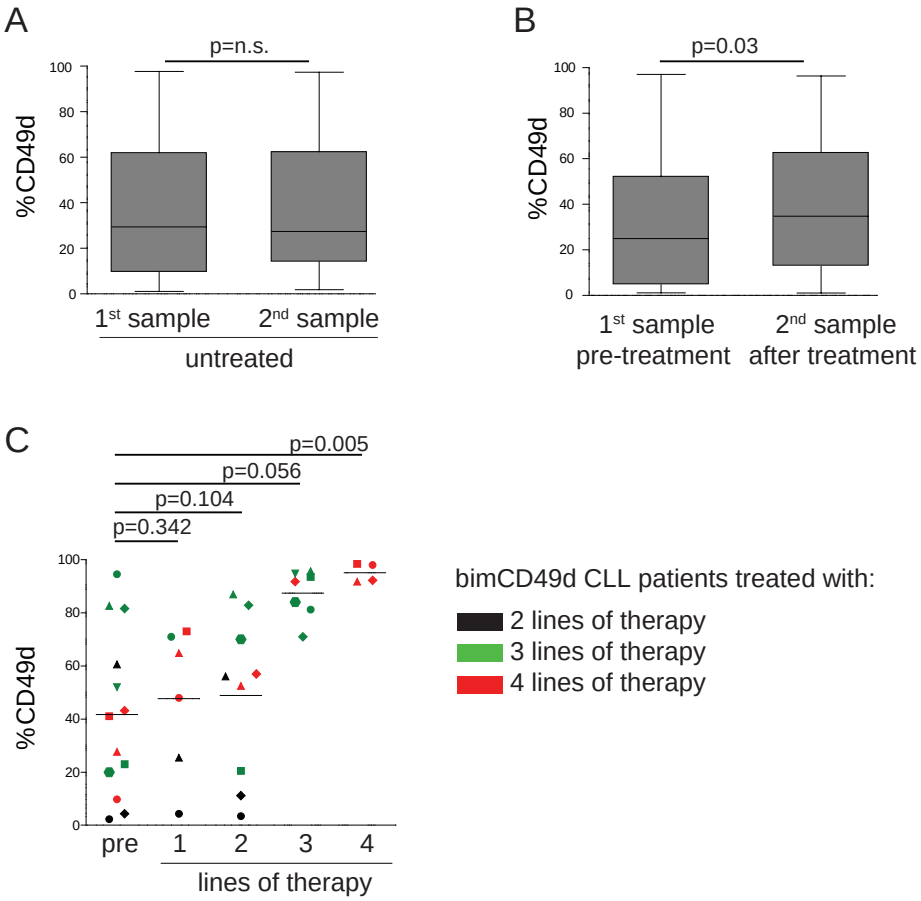

A
p=n.s.
%CD49d
100
80
60
40
20
0
1st sample
2nd sample
untreated
B
p=0.03
%CD49d
1st sample
pre-treatment
2nd sample
after treatment
C
p=0.005
p=0.056
p=0.104
p=0.342
%CD49d
pre
1
2
3
4
lines of therapy
bimCD49d CLL patients treated with:
2 lines of therapy
3 lines of therapy
4 lines of therapy

Figure 3

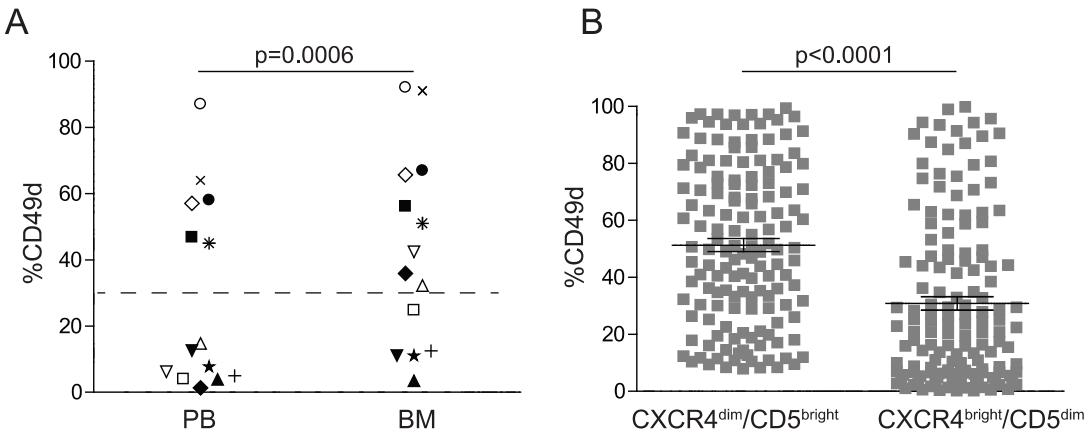

A
p=0.0006
100
80
60
40
20
0
%CD49d
PB
BM
B
p<0.0001
100
80
60
40
20
0
%CD49d
CXCR4dim/CD5bright
CXCR4bright/CD5dim

Figure 4

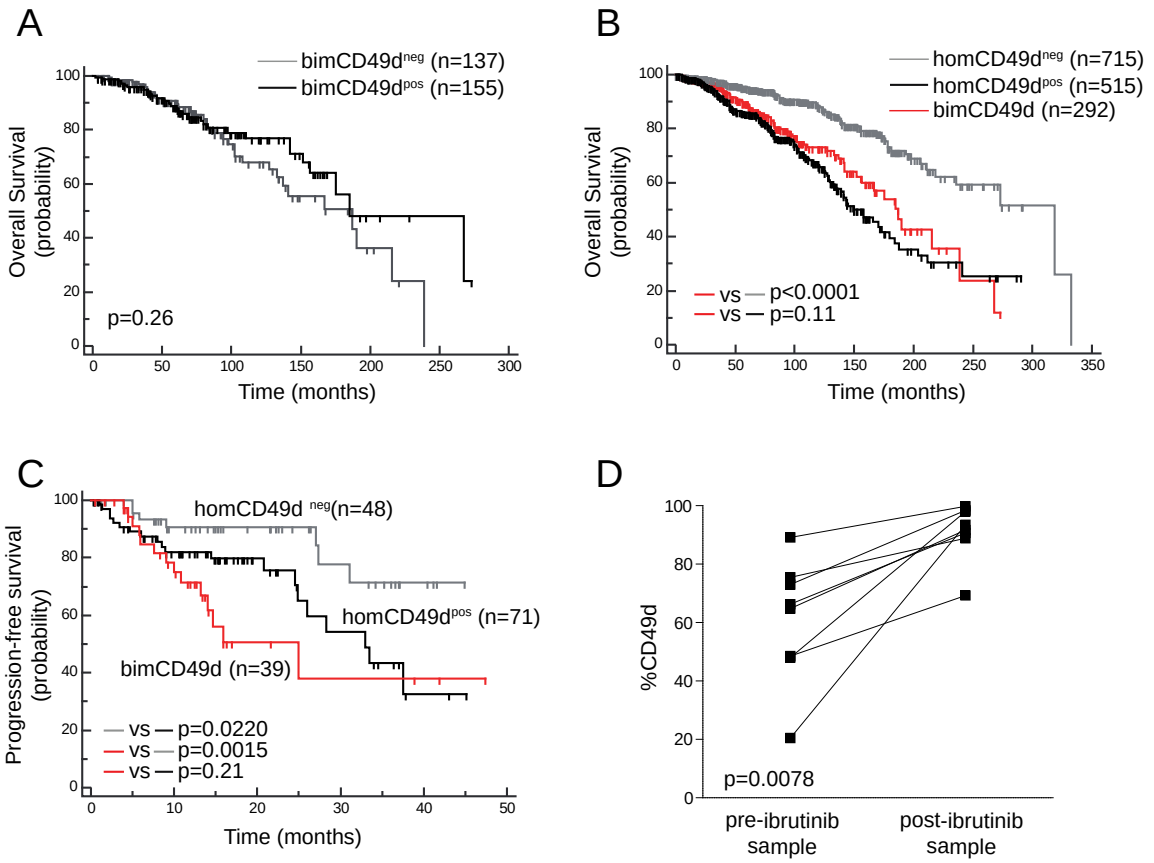
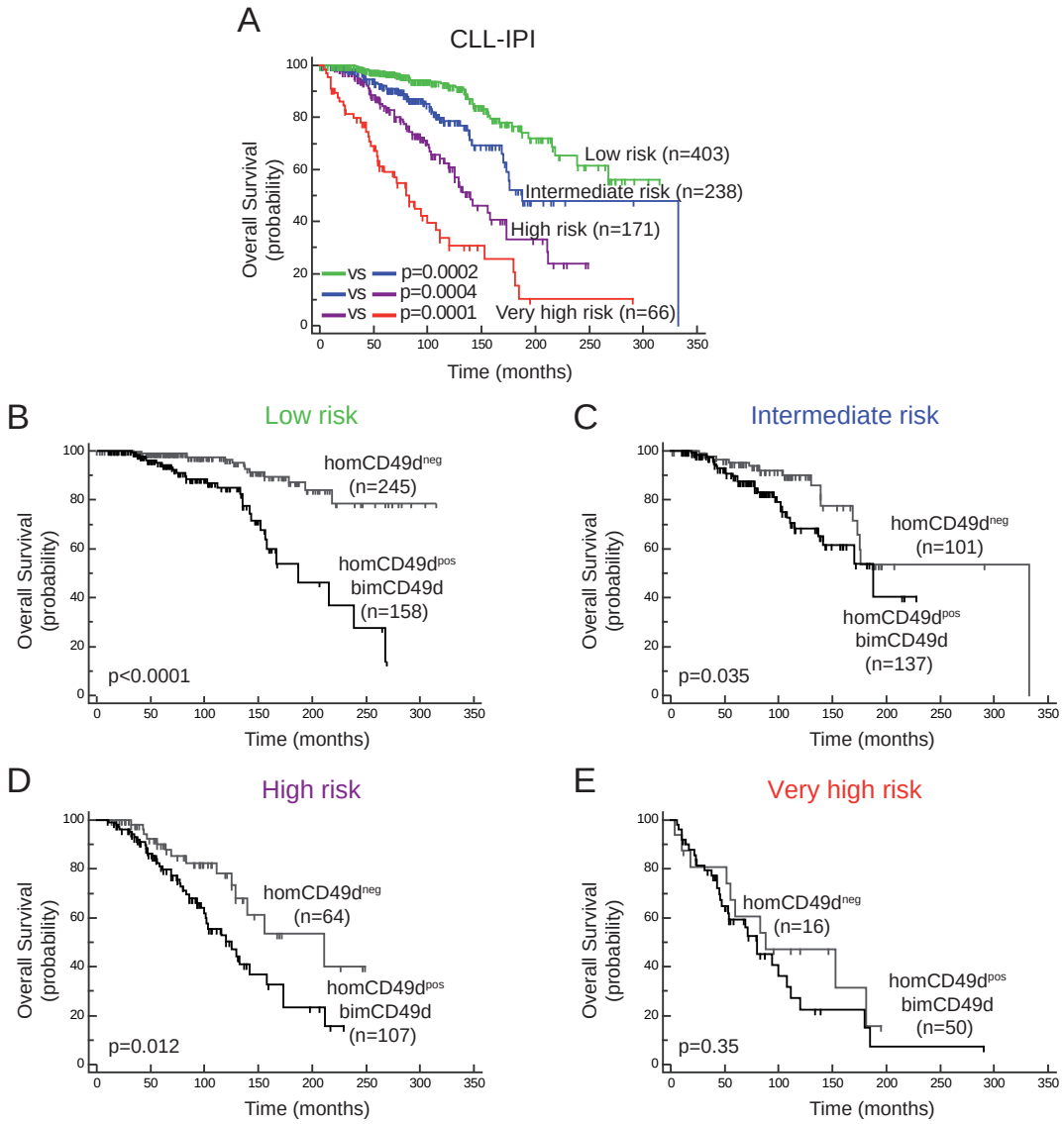

A
bimCD49d^neg (n=137)
bimCD49d^pos (n=155)
Overall Survival (probability)
p=0.26
Time (months)
B
homCD49d^neg (n=715)
homCD49d^pos (n=515)
bimCD49d (n=292)
Overall Survival (probability)
vs p<0.0001
vs p=0.11
Time (months)
C
homCD49d neg(n=48)
homCD49d^pos (n=71)
bimCD49d (n=39)
Progression-free survival (probability)
vs p=0.0220
vs p=0.0015
vs p=0.21
Time (months)
D
%CD49d
p=0.0078
pre-ibrutinib sample
post-ibrutinib sample

Figure 5


A
CLL-IPI
Overall Survival (probability)
Time (months)
Low risk (n=403)
Intermediate risk (n=238)
High risk (n=171)
Very high risk (n=66)
vs p=0.0002
vs p=0.0004
vs p=0.0001
B
Low risk
homCD49d^neg (n=245)
homCD49d^pos bimCD49d (n=158)
p<0.0001
C
Intermediate risk
homCD49d^neg (n=101)
homCD49d^pos bimCD49d (n=137)
p=0.035
D
High risk
homCD49d^neg (n=64)
homCD49d^pos bimCD49d (n=107)
p=0.012
E
Very high risk
homCD49d^neg (n=16)
homCD49d^pos bimCD49d (n=50)
p=0.35